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Use of Clove, Mint and Camphor Essential Oils on Confinement of Clown Anemonefish *Amphiprion ocellaris* (Cuvier 1830): Anesthetic Effects and Influence on Water Quality

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Abstract

The aim of this study was to evaluate the anesthetic efficacy of essential oils of clove, mint and camphor to clownfish *Amphiprion ocellaris* and their effects on water quality in simulated confinement transport conditions. Anesthetic effects of clove, mint and camphor oils were tested at concentrations of 2.5, 5.0 and 7.5 µL L^{-1}; 20, 25 and 30 µL L^{-1}; and 100, 120 and 140 µL L^{-1}, respectively. Confinement periods of 6 h, 12 h and 24 h were simulated (n=8 fish/time/concentration). Animals were randomly selected and transferred to polyethylene bags (16 × 30 cm, 5 fish L^{-1}). Water concentrations of dissolved oxygen (DO), nitrogen in the form of total ammonium (N-TA=NH_3+NH_4^+) and non-ionized ammonia (N-NH_3) and pH were measured before closing and after opening the bags. Different confinement densities of *A. ocellaris* (5, 10, 15 and 20 fish L^{-1}) were tested in polyethylene bags containing water and anesthetics essential oils. Concentrations of 5, 25 and 120 µL L^{-1} (clove, mint and, camphor oils, respectively) were used during 24 h of confinement. Water-quality parameters monitored were the same as in the previous experiment, with addition of measuring concentrations of dissolved CO_2 in water. The use of mint oil (25 µL L^{-1}, maximum density of 10 fish L^{-1}) significantly reduced the concentration of N-TA. At low densities (5 fish L^{-1}) clove (5 µL L^{-1}) and camphor (120 µL L^{-1}) oils can also be safely used for confinement of *Amphiprion ocellaris* for 24 h.

Keywords: Immersion anesthesia; Ornamental fish; Plant oils; Reef fish; Transportation

Introduction

Marine ornamental fish are captured and traded by at least 45 countries, located mainly in tropical and subtropical zones. Emerging countries are responsible for 63% of the total worldwide reef fish exportation [1]. Two third of this market are dominated by Philippines and Indonesia and the other remaining third is from countries such as the Maldives, Vietnam, Thailand, Sri Lanka, Puerto Rico, Australia, Hawaii and Brazil [2]. United States, European Union and Japan are the biggest importers of ornamental fish [3] and the clown anemonefish *Amphiprion ocellaris* is the most traded species in terms of number of individuals [4].

Due to the large geographic distances between collection and/or cultivation sites and trader establishment, and the fact that fish must arrive in good conditions at their destination, the productive chain of ornamental fish requires complex logistics for transportation and distribution. The conditions under which the fish are transported directly influence the health and viability of traded animals and, consequently, the economic the operation efficiency [5].

The greatest challenges during the transport of ornamental fish, especially when performed over long distances and at high densities, are to avoid excessive stress, mechanical shocks and deterioration of water quality caused by the elimination of metabolic waste from the fish body, mainly ammonia and carbon dioxide [6-8]. Because ornamental fish are packed in small volumes of water under transport conditions, organic wastes can be accumulated and reach internal toxic concentrations [9].

Sedation is one option used to minimize stress and possible injuries caused by the agitated state of fish during transport [10-12]. Anesthesia reduces metabolic rates and consequently decreases the uptake of oxygen and excretion of metabolic products into water throughout the transport period [13]. Selection of products to be used during transport often depends on factors such as availability, cost-effectiveness, facility of use and safety of handlers [14]. These factors make some essential oils potentially suitable for anesthesia of fish during confinement conditions of transport.

The clove *Eugenia caryophyllata* essential oil has been studied as a sedative to minimize the effects of transportation on marbled spinefoot *Siganus rivulatus* [15], largemouth bass *Micropterus salmoides* [10], mongolian redfin *Culter mongolicus* [8] and angelfish *Pterophyllum scalare* [16]. The mint *Mentha arvensis* and camphor *Cinnamomum camphora* essential oils have proven anesthetic effects on clownfish *Amphiprion ocellaris* and can be used during animal laboratory handling [17]. The aim of the present study was to evaluate the anesthetic efficacy of clove, mint and camphor essential oils on clown anemonefish *Amphiprion ocellaris* and their effects on water quality under confinement conditions similar to transport.

Material and Methods

Experiments were performed at the Integrated Group of Aquaculture and Environmental Studies (GIA) laboratorial facilities. GIA is located in Curitiba, Paraná, Brazil.

Animal care

Two hundred juveniles *A. ocellaris* were acquired from Azul Fish

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Farm™ (São Paulo, Brazil). Fish (Wt= 0.47 ± 0.42 g; Lt= 2.75 ± 0.39 cm) were transported at a density of 10 fish L^{-1}, in plastic bags containing water and oxygen (1:2 ratio). For transportation, plastic bags were placed into isothermal boxes. The trip (from packing until arrive) lasted 7 hours. In laboratory, animals underwent gradual acclimation of temperature, pH and salinity for approximately 30 min. Acclimated fish were transferred to three 200 L glass tanks. Black plastic film was attached to the back of the tanks to reduce external light incidence. A saltwater recirculation system connected to the tanks maintained water quality stable (salinity= 30 g L^{-1}, temperature= 24 ± 0.5 ºC; pH= 7.9 ± 0.02; nitrogen in the form of total ammonium ($N\text{-}TA=NH_3+NH_4^+ \leq$ 0.25 mg L^{-1}) and fish were kept in these conditions for 180 days. Partial water changes (25% v/v) were performed weekly. Fish were fed twice daily *ad libitum* with a commercial diet containing 47.5% of crude protein (TETRA™, MELLE, Germany). One hour after feeding leftover food and fecal particles were siphoned.

Anesthetic effect of essential oils to fish and its influence on water quality under confinement conditions

Three different concentrations of clove (2.5, 5.0 and 7.5 μL L^{-1}), mint (20, 25 and 30 μL L^{-1}) and camphor (100, 120 and 140 μL L^{-1}) essential oils were tested simulating confinement conditions typically used during transport. Confinement periods of 6 h, 12 h and 24 h (n=8 fish per time/concentration) were tested and concentrations were determined based on a previous study [17] and on a pilot test.

Essential oils were acquired from Ferquima™ Indústria e Comércio de Óleos Essenciais (São Paulo, Brazil). The essentials oils main compounds, obtained by chromatographic analysis, were: camphor oil= 1.8-cineole (35.5%), limonene (30.0%), alpha-pinene (13.0%) and para-cymene (10.0%); clove oil = eugenol (85.0%) and beta caryophyllene (13.0%); mint oil = l-menthol (37.0%), menthone (20.25%), limonene (6.75%), isomenthone (7.48%), menthyl acetate (4.60%), isopulegone (1.81%), pulegone (1.39%), carvone (0.08%) and cineole (0.34%). Stock solutions were prepared by diluting each essential oil in ethanol 100% (1:10). All results were compared to those obtained from a control group in which the fish were exposed only to clean saltwater.

To simulate confinement conditions comparable to those used for fish transport in Brazilian market, fish were randomly selected from the maintenance tanks and transferred to polyethylene bags (16 × 30 cm) at a density of 5 fish L^{-1}. Bags were filled with 400 mL of saltwater and pure oxygen (1:2 ratio) plus one of the experimental anesthetic concentrations. The containers were sealed with rubber bands and arranged in isothermal boxes (identical to those used to transport ornamental fish), and remained there for one of the tested confinement period.

At the end of the confinement exposure period, the anesthesia stage induced by each essential oil was evaluated. The considered stages of fish anesthesia were: (I) absence of reaction to touch and to visual stimulus; (II) initial loss of balance, characterized by difficulty to maintain normal swimming position; (III) total loss of balance, uncoordinated swimming; (IV) minimal opercula movement, no swimming and (V) medullar collapse and no opercula beating, death. For recovery fish were distributed evenly in a salt-water recirculation system with a 900 L capacity, divided into 15 storage tanks of 60 L. This system had controlled water quality conditions similar to the maintenance tanks. During recovery, fish were visually monitored for 72h in order to identify morality, appearance of body injuries and alterations on feeding behavior.

Physical-chemical water quality parameters were measured in two moments: (1) before the injection of oxygen and closing of the bags (initial values) and (2) after opening the packages (final values). Water samples were immediately tested for the determination of pH (AZ™ 86505 PHMETER, Taiwan), oxygen concentration and temperature (YSI™ 550A, USA). NA-T concentrations were spectrophotometrically (SPECTRONIC 20 GENESYS™, England) determined according to the indophenol method [18]. The concentration of N-TA and $N\text{-}NH_3$ were calculated according to the formula described by Ostrensky, Marchiori [19].

Effect of fish density on water quality using essential oils for anesthetic purposes

A second experiment was performed to evaluate the effect of clove, mint and camphor oils on water quality parameters during confinement condition at different densities. Each essential oil was tested in four densities (5, 10, 15 and 20 fish L^{-1}). These values were based on pilot experimental results. Experimental procedures consisted in placing five fish in one polyethylene bag (4 replicates/treatment). The total volume of saltwater was reduced to increase the density of fish in the confinement bag. For the densities of 5, 10, 15 and 20 fish L^{-1} were used 1.000, 500, 333 and 250 mL of clean saltwater, respectively. Based on the results obtained on the previously experiment, one concentration of each anesthetic oil was used in the density test: 5 μL L^{-1} of clove oil, 25 μL L^{-1} of mint oil and 120 μL L^{-1} of camphor oil. Results were compared with a control group (clean saltwater with no addition of anesthetic substances).

The methods used for housing animals in polyethylene bags and determining the physic-chemical parameters of the confinement water were similar to the previous experiment. In this experiment, the concentration of dissolved carbon dioxide (CO_2) in water was also measured by colorimetric titration using sodium hydroxide and phenolphthalein [20] before (initial) and after (final) 24 h. Initials levels of DO, CO_2, temperature and pH before the experiment were 5.89 ± 0.34 mg L^{-1}, 5.60 ± 0.49 mg L^{-1}, 24.5 ± 0.40 ºC and 7.9 ± 0.02 (mean ± standard deviation), respectively. Concentrations of N-TA and $N\text{-}NH_3$ remained below detection limit of the method used. Mortality and feeding behavior were also evaluated during the 72h immediately following the experiment.

Statistical analysis

Normality of data was assessed by Shapiro-Wilk test. Differences between tested variables were analyzed through Kruskal-Wallis test ($p<0.05$). The results obtained using each anesthetic in different concentrations, confinement times and densities were analyzed separately. Subsequently, the outcomes of different anesthetic treatments were compared in terms of confinement water quality parameters. All analyses were performed using the software Statsoft Statistica™ version 10.0.

Results

Anesthetic effect and influence of using essential oils on water quality under transport conditions

No mortally was observed during maintenance period. Initial level of DO, temperature and pH were 6.0 ± 0.18 mg L^{-1}, 24.5 ± 0.32°C and 7.9 ± 0.01, respectively. Concentrations of N-TA and $N\text{-}NH_3$ remained undetectable. Fish exposed to the lowest concentration of clove oil (2.5 μ L^{-1}) showed no characteristic behavioral signs of anesthesia. Other two tested concentrations (5 and 7.5 μL L^{-1}) induced evidences of anesthetic stage III and IV after 6 h of exposure and stages II and III after 12 h. All mint oil concentrations tested have induced anesthesia. At 6 h of

Treatment	Concentration (µL L^{-1})	Stage			Mortality (%)
		6 h	12 h	24 h	
Control	0	—	—	—	0
Clove	2.5	—	—	—	0
	5.0	III	II	—	0
	7.5	IV	III	—	50.0[a]
Mint	20	II	—	—	0
	25	III	II	—	0
	30	IV	IV	—	25.0[a]
Camphor	100	II	—	—	0
	120	IV	II	II	0
	140	IV	IV	II	37.5*

*Note: All deaths occurred on the 24 h of confinement treatments.

Table 1: Anesthetic stage and mortality (%) observed after *Amphiprion ocellaris* confinement for different periods of using different concentrations of clove, mint and camphor oils as anesthetic substances in addition to the control treatment (clean saltwater).

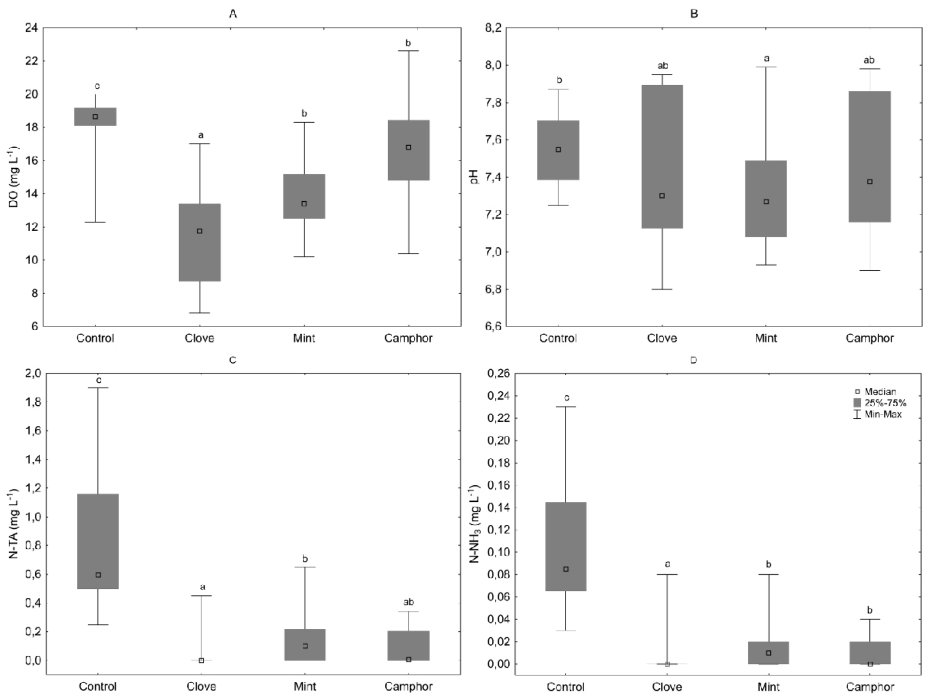

Figure 1: Measured values of dissolved oxygen (DO), pH, nitrogen in the form of total ammonium (N-TA=NH_3+NH_4^+) and non-ionized ammonia (N-NH_3) in confinement water using clove, mint, camphor oils as anesthetic and a control treatment to *Amphiprion ocellaris*. Letters indicate significant differences among treatments (p<0.05).

exposure, stages II, III and IV were observed in treatments 20, 25 and 30 µL L^{-1}, respectively. Fish exposed to the two highest concentrations of this same oil showed signs of anesthesia stage II and IV after 12 h, respectively. The lowest concentration of camphor oil (100 µL L^{-1}) was sufficient to induce only stage II at 6 h, while 120 and 140 µL L^{-1} induced stage IV at 6 h and led to stages II and IV, respectively, after 12 h (Table 1). No behavioral signs of anesthesia were identified in fish 24 h after starting the experiment at any tested concentration of clove and mint oils. In contrast, the two highest concentrations of camphor oil were sufficient to keep the animals at anesthetic stage II until the end of this period. Moreover, the highest concentrations tested for all oils caused the death of some animals, reaching 50% with clove oil. The deaths always occurred between 12 h and 24 h after initial exposure to anesthetic.

There were no significant effects on the water-quality parameters when comparing different concentration of the same oil and period. However, there were significant differences in all water-quality parameters among the different periods of confinement. For all treatments, there was a declining trend in the DO concentration in water relative to the control. This same pattern is evident when comparing pH values, through water acidification occurred over the 24 h of experimentation in all treatments. At the beginning of the experiment, there were no detectable ammonia concentrations in the control. During the experiment, ammonia levels reached up to 1.90 mg L^{-1} of N-TA and 0.23 mg L^{-1} of N-NH_3 in this same treatment. In treatments with camphor oil, there was an increase in the concentrations of N-TA and N-NH_3 after 12 h. However, these values remained visually unchanged into 24 h. In contrast, for the treatments with clove and mint oils, except at a concentration of 7.5 µL L^{-1}, which remained stable, there was an increase in the concentrations of N-TA and N-NH_3 at 6 h and a decreasing trend between 12 h and 24 h, when concentrations were near to zero.

Pooled analysis of the effects of each anesthetic on water quality

Water quality parameters (DO, pH, N-TA and N-NH_3) did not

Treatment	Concentration (μL L^{-1})	DO (mg L^{-1})			pH		
		6 h	12 h	24 h	6 h	12 h	24 h
Control	0	17.7 (12.3-18.9)[a]	18.8 (18.4-20.0)[b]	19.2 (18.3-20.0)[b]	7.83 (7.45-7.87)[a]	7.60 (7.50-7.60)[a]	7.34 (731-7.45)[b]
Clove oil	2.5	14.3 (13.7-16.6)	13.4 (12.3-14.0)	13.8 (11.5-14.0)	7.92 (7.91-7.92)[a]	7.37 (7.247.63)[ab]	7.18 (7.08-7.24)[b]
	5.0	12.4 (6.8-12.8)[a]	11.7 (6.9-8.4)[a]	7.4 (6.9-8.4)[b]	7.91 (7.89-7.93)[a]	7.28 (7.26-7.30)[ab]	6.86 (6.83-6.88)[b]
	7.5	10.5 (9.1-11.3)[a]	11.7 (10.9-12.2)[a]	7.3 (7.2-7.8)[b]	7.91 (7.86-7.94)[a]	7.32 (7.28-7.41)[ab]	6.87 (6.80-6.92)[b]
Mint oil	20.0	16.3 (15.8-16.8)[a]	13.1 (14.5-18.7)[b]	15.1 (14.1-16.9)[a]	7.90 (7.87-7.99)[a]	7.13 (7.07-7.21)[b]	7.28 (7.27-7.32)[ab]
	25.0	15.2 (13.4-16.2)[a]	12.1 (10.4-19.6)[a]	11.7 (11.2-12.9)[b]	7.93 (7.81-7.99)[a]	7.24 (7.08-7.34)[ab]	6.97 (6.98-7.00)[b]
	30.0	14.2 (13.4-15.2)[a]	13.6 (12.9-15.5) [a]	11.4 (10.2-12.2) [b]	7.43 (7.38-7.49)[a]	7.21 (7.17-7.30) [ab]	6.99 (6.98-7.00)[b]
Camphor oil	100.0	17.6 (16.6-19.3)[a]	16.8 (14.5-18.7)[ab]	14.2 (10.5-17.2)[b]	7.81 (7.53-7.87)[a]	7.40 (7.34-7.43)[ab]	7.01 (6.90-7.15)[b]
	120.0	18.5 (17.6-22.6)[a]	17.0 (10.4-19.6)[ab]	14.3 (13.6-17.4)[b]	7.89 (7.87-7.89)[a]	7.39 (7.37-7.41)[ab]	7.11 (7.07-7.16)[b]
	140.0	18.5 (18.0-19.1)[a]	14.90 (12.9-15.5)[b]	16.0 (15.1-17.3)[ab]	7.88 (7.87-7.98)[a]	7.21 (7.18-7.33)[b]	7.17 (7.11-7.20)[b]

Note: Letters indicate statistical difference ($p<0.05$) among tested confinement periods (horizontal). There were no statistical differences ($p>0.05$) among treatments in the same confinement period (vertical) ($p>0.05$).

Table 2: Median (min and max) values of dissolved oxygen (DO) and pH in saltwater after different periods of *Amphiprion ocellaris* confinement using the clove, mint and camphor oils as anesthetic substances and a control treatment.

Treatment	Concentration (μL L^{-1})	N-TA (mg L^{-1})			$N\text{-}NH_3$ (mg L^{-1})		
		6 h	12 h	24 h	6 h	12 h	24 h
Control	0	0.73 (0.25-1.28)[ab]	0.50 (0.49-0.50)[a]	1.20 (0.30-1.90)[b]	0.12 (0.04-0.16)[ab]	0.07 (0.07)[a]	0.13 (0.06-0.23)[b]
Clove oil	2.5	0.27 (0.00-0.41)[a]	0.00 (0.00)[b]	0.00 (0.00)[b]	0.05 (0.00-0.08)[a]	0.00 (0.00)[b]	0.00 (0.00)[b]
	5.0	0.22 (0.00-0.45)[a]	0.00 (0.00)[b]	0.00 (0.00)[b]	0.03 (0.00-0.08)[a]	0.00 (0.00)[b]	0.00 (0.00)[b]
	7.5	0.00 (0.00-0.13)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00-0.02)	0.00 (0.00)	0.00 (0.00)
Mint oil	20.0	0.10 (0.09-0.10)[ab]	0.22 (0.18-039)[a]	0.00 (0.00)[b]	0.01 (0.01)[a]	0.01 90.01-0.03)[a]	0.00 (0.00)[a]
	25.0	0.21 (0.10-0.45)[a]	0.21 (0.18-0.43)[a]	0.00 (0.00)[b]	0.03 (0.01-0.08)[a]	0.02 (0.01-0.04)[a]	0.00 (0.00)[b]
	30.0	0.24 (0.10-0.65)[a]	0.14 (0.03-0.20)[a]	0.00 (0.00)[b]	0.02 (0.01-0.08)[a]	0.01 (0.00-0.02)[b]	0.00 (0.00)[b]
Camphor oil	100.0	0.00 (0.00)[a]	0.11 (0.01-0.23)[b]	0.11 (0.00-0.32)[b]	0.00 (0.00)	0.01 (0.00-0.03)	0.01 (0.00-0.03)
	120.0	0.00 (0.00)[a]	0.20 (0.01-0.34)[b]	0.20 (0.10-0.23)[b]	0.00 (0.00)[a]	0.02 (0.01-0.03)[b]	0.02 90.01-0.02)[b]
	140.0	0.00 (0.00)[a]	0.18 (0.07-0.32)[b]	0.00 (0.00)[a]	0.00 (0.00)[a]	0.02 (0.00-0.04)[b]	0.00 (0.00)[a]

Note: Letters indicate statistical difference ($p<0.05$) among tested confinement periods (horizontal). There were no statistical differences ($p>0.05$) among treatments in the same confinement period (vertical) ($p>0.05$).

Table 3: Median (min and max) values of nitrogen in the form of total ammonium ($N\text{-}TA=NH_3+NH_4^+$) and non-ionized ammonia ($N\text{-}NH_3$) concentrations after different periods of *Amphiprion ocellaris* confinement using clove, mint and camphor oils as anesthetic and a control group.

presented statistical differences separately ($p>0.05$). After, these data were analyzed pooling all of the experimentation periods, and showed some significant differences caused by the anesthetics tested. As seen in Figure 1, the use of anesthetics caused DO concentrations in transport water to decrease by at least 10%, reaching 16.71 mg L^{-1} (measured in the camphor oil treatment). The lowest median value, 11.88 mg L^{-1}, was recorded in the clove oil treatment; this value was 56% higher in the control, reaching 18.57 mg L^{-1}. The treatments with anesthetics featured greater variability in pH values (which ranged between 6.8 and 8.0) than the control (from 7.24 to 7.84). However, the greatest difference was observed in the concentrations of N-TA and $N\text{-}NH_3$. In this case, the medians measured for the control (0.61 and 0.08 mg/l of N-TA and $N\text{-}NH_3$, respectively) were significantly higher than those measured for treatments with clove (both medians were 0.00), mint (medians of 0.09 and 0.01) and camphor (medians of 0.02 and 0.00) oils.

Effects of different fish densities on water quality during simulated confinement conditions

Increasing the density of fish tended to increase the final measured concentrations of CO_2 and DO in practically all of the treatments including the control (Table 2). There was an opposite trend for pH: the values decreased with greater densities. The largest differences in median pH were measured in the control (0.27), while these differences ranged between 0.11 and 0.18 for the other treatments. Concentrations of N-TA and $N\text{-}NH_3$ measured in the control and in the treatments containing clove and camphor oils showed a significant increase beyond the density of 10 fish L^{-1}. When using mint oil, such increase only occurred at densities starting from 15 fish L^{-1} (Table 3).

Pooled analysis of data obtained during the simulated confinement in different animal densities

The final DO concentrations recorded in the control were 29 to 42% higher than those measured in treatments containing anesthetics. In absolute terms, while the median DO in the control was 16.50 mg L^{-1}, the concentration for treatment containing mint oil was 11.65 mg L^{-1} (Figure 2). The CO_2 concentrations of the control and camphor oil treatments were 10 mg L^{-1}, which is higher than the values in clove (8.0 mg L^{-1}) and mint (8.5 mg L^{-1}) oils treatments.

The concentration (median) of N-TA in the control was 3.23 mg L^{-1}, which was significantly higher than those observed in the treatments containing anesthetic oils. A similar pattern was observed for $N\text{-}NH_3$. The $N\text{-}NH_3$ concentration was 41%, 140% and 200% higher in the control (median 0.24 mg L^{-1}) than the median concentrations obtained in treatments containing clove, mint and camphor oils, respectively. The pH results can be split into two groups: one including the control (median 7.03) and mint oil (median 7.00) and another group with

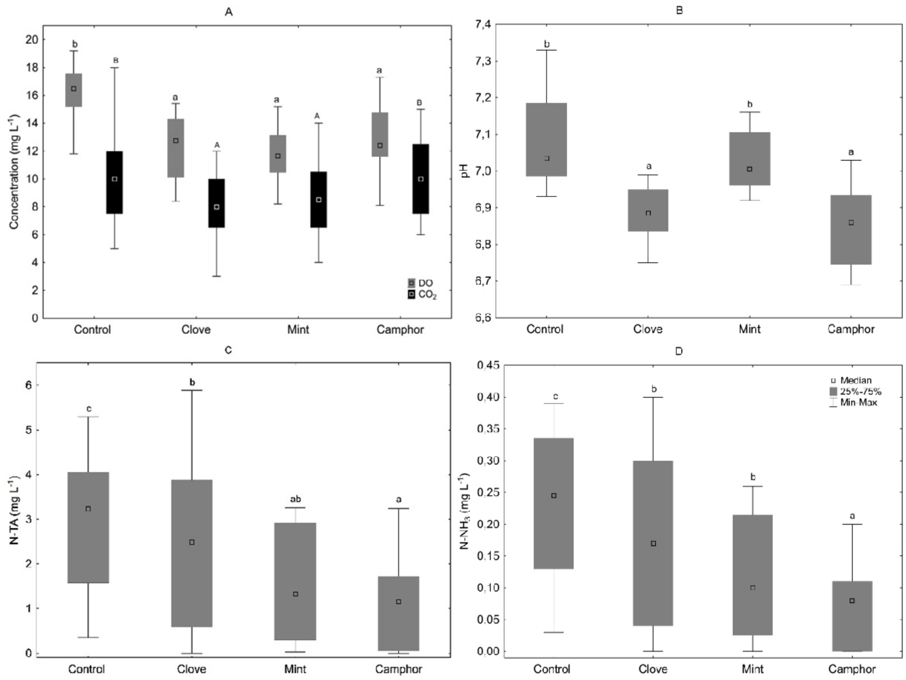

Figure 2: (A) Range of concentrations of dissolved oxygen (DO) and carbon dioxide (CO_2), (B) nitrogen in the form of total ammonium (N-TA=NH_3+NH_4+) and non-ionized ammonia (N-NH_3) and (C) pH in transport water using clove, mint and camphor oils or no anesthetic (control). Different letters indicate significant differences among treatments ($p<0.05$).

Treatment	Density Fish L^{-1}	DO (mg L^{-1})	CO_2 (mg L^{-1})	N – TA (mg L^{-1})	N-NH_3 (mg L^{-1})	pH
Control	05	14.00 ^{a} (11.8 – 15.0)	05.0 ^{a} (5.0 – 8.0)	0.57 ^{a} (0.35 – 1.01)	0.06 ^{a} (0.03 – 0.10)	7.24 ^{a} (7.24 – 7.33)
	10	16.90 ^{b} (15.4- 17.8)	09.5 ^{b} (7.0 – 12.0)	2.59 ^{b} (2.15 – 2.73)	0.19 ^{b} (0.16 – 0.20)	6.97 ^{b} (6.93 – 6.98)
	15	17.10 ^{b} (16.0 – 17.6)	11.0 ^{bc} (10.0 – 12.0)	4.05 ^{c} (3.74 – 4.71)	0.31 ^{c} (0.30 – 0.36)	7.01 ^{c} (7.00 – 7.05)
	20	17.70 ^{b} (16.3 – 19.2)	13.5 ^{c} (11.0 – 18.0)	4.29 ^{c} (3.79 – 5.29)	0.36 ^{c} (0.29 – 0.39)	7.09 ^{c} (6.99 – 7.15)
Clove (5 µL L^{-1})	05	10.15 (9.4 – 13.3)	07.0 ^{a} (7.0 – 9.0)	0.00 ^{a} (0.00)	0.00 ^{a} (0.00)	6.97 ^{a} (6.83 – 6.99)
	10	12.95 (8.4 – 15.2)	08.0 ^{a} (6.0 – 9.0)	3.01 ^{b} (1.18 – 4.25)	0.22 ^{b} (0.08 – 0.31)	6.92 ^{a} (6.90 – 6.93)
	15	12.80 (9.1 – 15.4)	08.0 ^{ab} (6.0 – 12.0)	2.48 ^{b} (2.01 – 5.89)	0.17 ^{b} (0.13 – 0.40)	6.81 ^{b} (6.75 – 6.95)
	20	12.70 (10.4 – 14.3)	10.0 ^{b} (8.0 – 12.0)	3.72 ^{b} (3.49 – 4.70)	0.27 ^{b} (0.25 – 0.34)	6.86 ^{b} (6.84 – 6.87)
Mint (25 µL L^{-1})	05	10.90 ^{a} (9.2 – 12.6)	05.0 ^{a} (4.0 – 7.0)	0.29 ^{a} (0.03 – 0.43)	0.02 ^{a} (0 – 0.04)	7.13 ^{a} (7.11 – 7.16)
	10	09.55 ^{a} (8.2 – 12.1)	10.0 ^{bc} (8.0 -10.0)	0.28 ^{a} (0.19 – 0.45)	0.02 ^{a} (0.01 – 0.03)	6.95 ^{b} (6.92 – 6.96)
	15	13.40 ^{b} (10.9 – 15.2)	07.5 ^{b} (6.0 – 14.0)	2.61 ^{b} (2.22 – 3.25)	0.22 ^{b} (0.19 – 0.26)	7.05 ^{c} (7.02 – 7.10)
	20	13.15 ^{b} (11.2 – 15.2)	11.5 ^{c} (9.0 – 14.0)	3.03 ^{b} (2.21 – 3.17)	0.22 ^{b} (0.16 – 0.23)	6.97 ^{b} (6.94 – 6.98)
Camphor (120 µL L^{-1})	05	10.35 ^{a} (8.1 – 11.4)	07.0 ^{a} (6.0 – 8.0)	0.02 ^{a} (0 – 1.46)	0.00 ^{a} (0 – 0.09)	6.84 ^{a} (6.74 – 6.98)
	10	12.25 ^{b} (12.0 - 17.3)	09.0 ^{a} (7.0 -11.0)	1.16 ^{b} (0.42 – 1.98)	0.08 ^{b} (0.03 – 0.14)	6.98 ^{b} (6.91 – 7.03)
	15	13.10 ^{b} (11.8 – 14.8)	12.0 ^{b} (10.0 – 14.0)	1.88 ^{b} (1.28 – 3.24)	0.12 ^{b} (0.08 – 0.20)	6.86 ^{a} (6.81 – 6.87)
	20	14.80 ^{b} (12.6 – 15.6)	13.0 ^{b} (10.0 – 15.0)	1.89 ^{b} (0.04 – 2.35)	0.13 ^{b} (0 – 0.13)	6.73 ^{a} (6.73 – 6.75)

Note: Letters indicate difference among tested densities (vertical) ($p<0.05$).

Table 4: Concentrations (median, min. and max.) of dissolved oxygen (DO), dissolved carbon dioxide (CO_2), total ammonium (N-TA) and non-ionized ammonia (N-NH_3) and pH of water at 24 h after confinement using different densities of *Amphiprion ocellaris* combined with clove, mint and camphor oils or no anesthetic (control).

lower values including the treatments with clove and camphor oils (medians of 6.88 and 6.86, respectively) (Table 4).

Discussion

Establishing the concentration of a particular anesthetic to be used during the transport of reef fish is a very important and difficult task. If the anesthesia is too superficial, its effects can be null. However, if the anesthesia is too deep and causes a total loss of balance, the animals may sink and overlap at the bottom of the container, what, according to Coyle, Durborow [11], could lead to suffocation at high fish densities. Death can also occur due to poisoning effect caused by long exposure to the anesthetic itself. The substance's toxicity is associated with the duration of exposure, and its margin of safety decreases as the period of confinement increases [13,21]. Advanced stages of anesthesia involve reduced respiratory rate, leading to a reduction in blood O_2 levels and a concomitant increase of CO_2 levels, causing hypoxia [22]. In most cases, maintaining stage IV anesthesia for long periods without gill irrigation can result in death [23]. A combination of these factors could be responsible for the mortality that occurred in this study when using the highest concentrations of anesthetics.

Thus, among the tested concentrations, the intermediate ones (5, 25 and 120 μL L^{-1} of clove, mint and camphor oils, respectively) are the most appropriate for use in confinement for transportation of *A. ocellaris*. Although these concentrations induced stage III anesthesia (total loss of balance) during the first hours of transportation, they presented no deleterious effects to the fish. At these concentrations, the animals remained in stage II anesthesia (desirable) for most of the confinement transport period. In other words, the fish were subjected to a relatively mild degree of anesthesia that was still sufficient to safely mitigate the effects of adverse transport conditions.

These results indicate a clear influence of the duration of confinement transport on deterioration of water quality. In this regard, there were advantages and disadvantages of using anesthetics. The major advantage concerns the tendency to keep ammonia concentrations at low and safety levels over time. In the absence of anesthetics, animals maintained their normal metabolism and excreted more ammonia into the water. Because pH remained at proportionately higher in control compared to the treatments, especially clove and camphor oils, the concentrations of gaseous ammonia were significantly higher in the control. Because gaseous ammonia is known to be toxic to both fresh and saltwater fish [24,25] and other aquatic organisms [26], the use of anesthetics can potentially reduce the risk of animal death due accumulating toxic concentrations of nitrogenous wastes during confinement transport. In the control, N-NH_3 concentrations were recorded above 0.05 mg L^{-1}, which is considered the acceptable limit for marine fish [27], thus supporting this claim.

One disadvantage is a tendency for greater variability on pH range during the use of anesthetics. However, the pH gradient observed in the present study seems to have been insufficient to cause death or harm to animals. According to Chow, Chen [28], *A. ocellaris* can withstand pH up to 6.3 under transport conditions, which is below the minimum value measured for all tested treatments. Mint oil allowed 10 fish L^{-1} to be transported without deleterious changes in water-quality parameters. In treatments containing clove and camphor oils, N-TA and N-NH_3 concentrations increased at this same density, indicating the lower efficacy of these oils at maintaining water quality at high densities. As expected, CO_2 increased and pH consequently decreased at higher densities. However, there was also an increase in DO concentrations in all treatments over the duration of the experiment. According to Pramod, Sajeevan [12], elevated CO_2 concentrations reduce the capacity of hemoglobin to transport oxygen. Upon placing fish into an anesthetic solution, large amounts of mucus are secreted into the water and can accumulate in gills, thereby impairing gas exchange and consequently oxygen uptake. This impairment in the mechanism of gas exchange was also reported by Chow, Chen [28] and Pandit and Ghosh [29]. Studies involving the use of anesthetics during transport have also reported decreased oxygen uptake by guppy *Poecilia reticulata* [30], platy *Xiphophorus maculatus* [31] and angelfish [16]. The DO concentrations measured after 6 h, 12 h and 24 h were higher than the baseline in all tested treatments. This ideal confinement scenario is probably an association result from irrelevant oxygen consumption by fish under anesthetic influence and a high diffusion rate from pure oxygen atmosphere, created prior of the experimental beginning, to the water.

Conclusion

The use of essential oils for anesthetic purpose can be suggested as a viable way to reduce deleterious effects of confinement during transport of *A. ocellaris*. The results demonstrated the efficacy to use of mint oil (25 μL L^{-1}) and to safely transport this species at density of 10 fish L^{-1}. Clove (5 μL L^{-1}) and camphor (120 μL L^{-1}) oils could also be used at lower densities (5 fish L^{-1}) to sedate fish during 24 h of confinement. It is also recommended the evaluation of the essential oils use as anesthetic substances associated with buffering substances and ammonia removers during *A. ocellaris* confinement and transportation.

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